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Multiple discrete sites for premature RNA chain termination late in adenovirus-2 infection: Enhancement by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole

(adenovirus-2 transcriptional control/HeLa cells)

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ABSTRACT Discrete-sized short RNA chains that contain the distinctive oligonucleotides, including the 5'-capped oligonucleotide, characteristic of the first 600 nucleotides of the adenovirus type 2 (Ad-2) large, late, rightward-reading transcriptional unit (*16.4–99*) accumulate in Ad-2-infected HeLa cells. In the presence of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole the accumulation of these chains is enhanced, as is the accumulation of short chains from a neighboring rightward-reading transcriptional unit (*3.0–10.7*). These short chains appear to represent prematurely terminated transcripts. Late in infection there is a marked increase in RNA synthesis, *including* that of prematurely terminated short chains, from the large late transcriptional unit. This suggests that the increase in transcription and mRNA production from this region late in infection is not due to reduced "attenuation" of RNA synthesis.

Late in infection of HeLa cells by adenovirus (adenovirus type 2, Ad-2), RNA polymerase II (1, 2) copies a single large transcriptional unit to produce the primary RNA transcripts that are processed into approximately 14 different virus-specific mRNA molecules (3–11). The location of the RNA initiation site at *16.4*† on the physical genome has made possible an examination of whether each polymerase that starts transcription progresses all the way to the same termination site or region (4, 12, 13). From analysis of pulse-labeled RNA, both from infected cells labeled *in vivo* (5, 14) and from isolated nuclei labeled *in vitro* (15), it was found that roughly from a 4- to 6-fold greater molar amount of RNA was synthesized from the first 2000 nucleotides of this transcriptional unit than from any other region. However, it appears that once a polymerase passes the first ≈2000 base pairs it always continues to the end of the transcriptional unit near *99* (8, 13), even though the mRNA to be formed from any particular transcriptional event might derive from sequences thousands of bases upstream from the termination region.

An additional interesting aspect of the apparent premature termination of Ad-2-specific RNA chains was discovered while studying the mode of action of the RNA synthesis inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). This drug was first thought to be an inhibitor of RNA chain initiation (16–18). However, short chains of RNA made by RNA polymerase II (19) continued to be labeled after DRB treatment. Also in the presence of DRB there is continued synthesis of short RNA chains complementary to regions of the Ad-2 genome containing start sites for both late (14) and early RNA synthesis (20). DRB, however, inhibited the synthesis of Ad-2 RNA downstream from the first 400–800 nucleotides (14, 20).

In this paper we describe the oligonucleotide composition of short Ad-2-specific RNA that accumulated late in infection in the presence or in the absence of DRB and conclude that most of the prematurely terminated RNA chains start at the same site (*16.4*) as the long RNA chains that eventually serve as mRNA precursors (12, 21). Furthermore, premature RNA chain termination appeared to take place at multiple discrete loci. Finally, it was found that the prematurely terminated chains characteristic of the large late transcriptional unit were not synthesized equally rapidly, if at all, early in virus infection. Therefore the transition from early to late virus-specific nuclear RNA synthesis and mRNA production does not depend simply on the partial relief of a previously absolute premature chain termination but appears to depend on an increase in the initiation of RNA synthesis at *16.4*.

MATERIALS AND METHODS

The procedures used for cell and virus growth, DNA preparation, cell labeling, and labeled RNA preparation (14, 20, 22), as well as the Southern "blotting" technique for DNA transfer to nitrocellulose (23), electrophoresis, and fingerprinting of RNA (24, 25) were all described previously.

DRB was a gift from Arthur F. Wagner, Merck, Sharp & Dohme, and was dissolved in serum- and phosphate-free medium by stirring overnight at 37°C.

RESULTS

Promoter-proximal discrete, "short" RNA chains

In order to prepare ^{32}P-labeled Ad-2-specific short nuclear RNA for examination of size and oligonucleotide composition of the prematurely terminated chains, HeLa cells infected for 13 hr were collected and labeled in the presence or absence of DRB with $^{32}PO_4$ for 4–5 hr. The nuclear RNA was extracted and the 4–14S RNA was collected after sucrose gradient separation. This short RNA would be expected to contain any of the previously described prematurely terminated labeled RNA that accumulated during the labeling period (5, 14, 15). Samples of this short RNA from DRB-treated or untreated cells were hybridized to the Ad-2 DNA restriction fragment *Sma*-F, *11.1–18.2*, that contains the start site for the major late transcription unit and the eluted samples were examined in several ways.

(*i*) **Polyacrylamide Gel Electrophoresis.** The *11.1–18.2*-specific RNA from several experiments was subjected to gel electrophoresis in comparison with unpurified RNA. Large

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Abbreviations: Ad-2, adenovirus type 2; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.

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† The linear Ad-2 DNA is divided into 100 units of 350 bases each with 0 on the left end of the rightward reading strand (3).

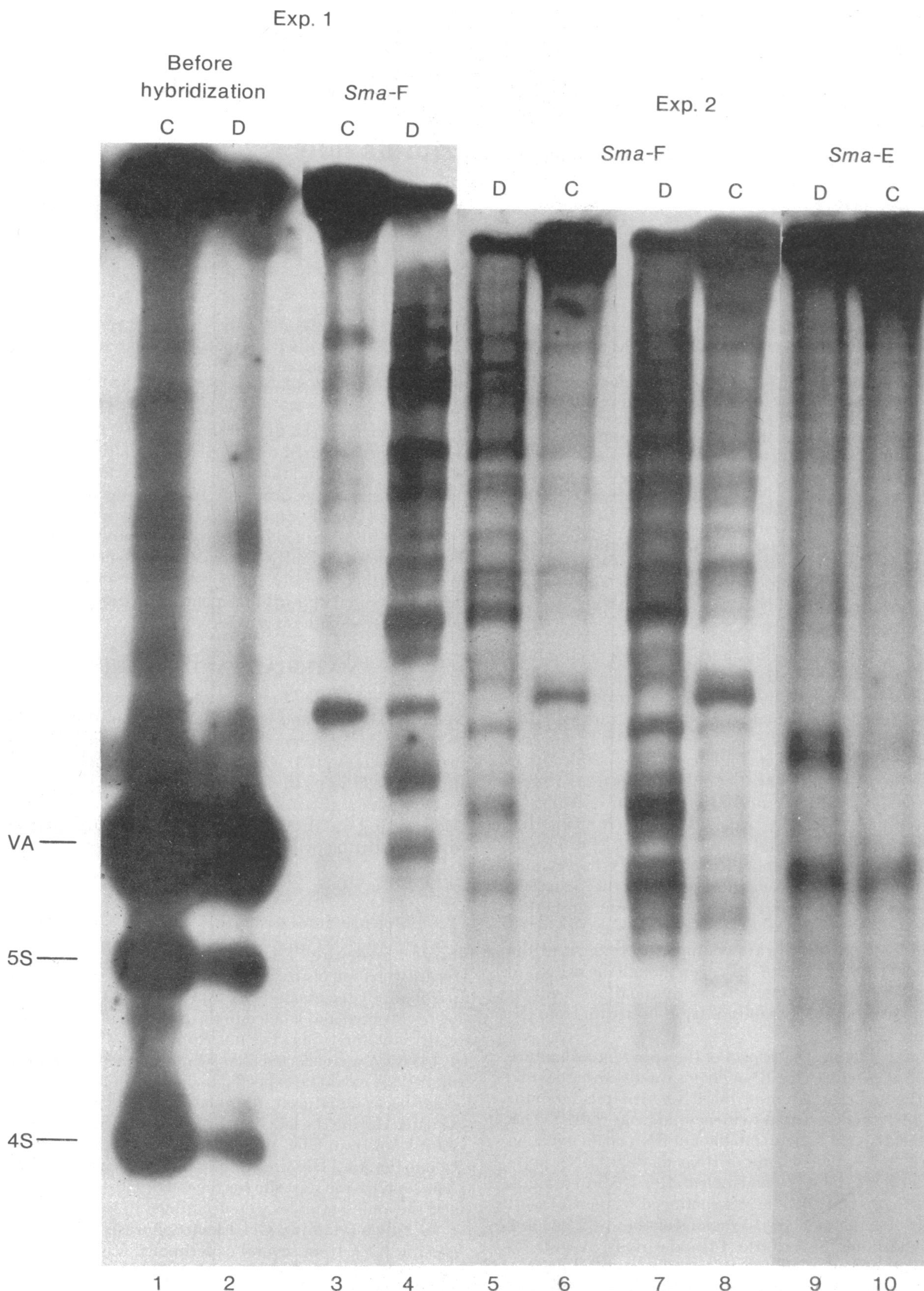

FIG. 1. Polyacrylamide gel electrophoretic analysis of DRB-resistant Ad-2-specific nuclear RNA. Ad-2-infected HeLa cells (2×10^8 per sample in 50-ml culture) 13–13.5 hr after the beginning of infection were labeled with $^{32}PO_4$ (50 mCi per culture; 1 Ci = 3.7×10^{10} becquerels) for another 4.5–5 hr. Samples were labeled without drug treatment (control lanes C) or after a 30- to 60-min pretreatment with 100 μM DRB (lanes D). The drug remained in the culture throughout the labeling period. (Identical results were obtained in the presence or absence of cycloheximide during the labeling period, a test that was made because "early" RNA synthesis is frequently studied in this manner.) The nuclear RNA preparations were treated with DNase (100 μg/ml), and the RNA was extracted and sedimented through a 15–30% sucrose gradient in a Beckman SW 27 rotor so that 18S marker RNA was close to the bottom of the tube and the 4S marker clearly was resolved from DNA at the top of gradient (14, 20). RNA between ≈4S and ≈14S was pooled, hybridized on nitrocellulose filters containing *Sma*-F (*11.1–18.2*) and *Sma*-E (*3.0–10.7*) DNA

numbers of specific RNA bands in the size range of 60–800 nucleotides were present in both the DRB-treated and the untreated control samples (Fig. 1). The RNA bands appeared to be reproducible in different experiments and were more prominent in preparations from DRB-treated cells. The strongest band in the control samples was ≈200 nucleotides long [estimated in experiment 1 by comparison during gel electrophoresis with VA RNA (26, 27), 160 nucleotides; 5S RNA (28), 121 nucleotides; and tRNA ≈80 nucleotides] and much less of the selected RNA was shorter than 200 nucleotides. In the DRB-treated samples the shorter bands were in general more intense and in both experiments very strong bands were seen in the ≈160- and ≈185-nucleotide regions (Fig. 1). (The size markers for the second experiment are not shown in the figure, but each of the bands migrated somewhat further in experiment 2 than in experiment 1).

A portion of the short 4–14S RNA was also hybridized to *Sma*-E (*3.0–10.7*) and the eluted RNA revealed, after electrophoresis and radioautography, two prominent short bands about 150 and 200 nucleotides in length and several other fainter bands. Again the DRB sample contained more of these short *Sma*-E specific RNA species than the control preparation.

(*ii*) **Rehybridization to Ad-2 DNA.** RNA initially selected on DNA filters bearing DNA from *11.1–18.2* or *3.0–10.7* on the genome was rehybridized to Ad-2 DNA that had been digested with either *Hin*dIII or *Kpn* I endonuclease, separated by electrophoresis, and attached to nitrocellulose filters by the Southern technique (23). The originally purified RNA samples contained the expected sequences: the RNA from both DRB-free and DRB-treated cells originally selected on *11.1–18.2* DNA hybridized strongly to both the *Hin*dIII C fragment (*8.0–17.0*) and the *Hin*dIII B fragment (*17.0–31.5*) but only slightly to other fragments. Similarly, RNA from both control and DRB-treated cells originally selected on *3.0–10.7* DNA hybridized strongly to both the *Hin*dIII A fragment (*0.0–8.0*) and the *Hin*dIII C fragment (*8.0–17.0*) but only slightly to other fragments.

(*iii*) **Poly(U)-Sepharose Chromatography.** The short RNA selected by hybridization to *11.1–18.2* DNA was passed through a poly(U)-Sepharose column. When the bound fraction was subjected to polyacrylamide gel electrophoresis and autoradiography, no poly(A)-containing RNA band was detected. In addition, Ziff and Evans (12) have reported that there is no RNA in the cytoplasm with the oligonucleotide composition of the *11.1–18.2*-specific RNA except for the cap sequences.

(*iv*) **Fingerprint Analysis and Detection of "Caps."** A more precise localization of the start sites for the short nuclear RNA was available by comparing the two-dimensional oligonucleotide patterns of the *11.1–18.2* short RNA with the pattern known to exist in longer nuclear molecules. The latter pattern had been confirmed by direct sequence analysis of the DNA (12). Short *11.1–18.2*-specific nuclear RNA from both DRB-treated and control cells was prepared and analyzed by two-dimensional fingerprinting. The fingerprint of the DRB-resistant material (Fig. 2) was found to be very similar if not identical to the fingerprint reported for the 5′ end of the large Ad-2 transcript (12). In particular, there are five large distinctive oligonucleotides that result from T1 digestion of RNA transcribed from the first ≈200 nucleotides of the large late transcription unit. All these oligonucleotides appeared present in approximately equal amounts in the DRB-resistant short

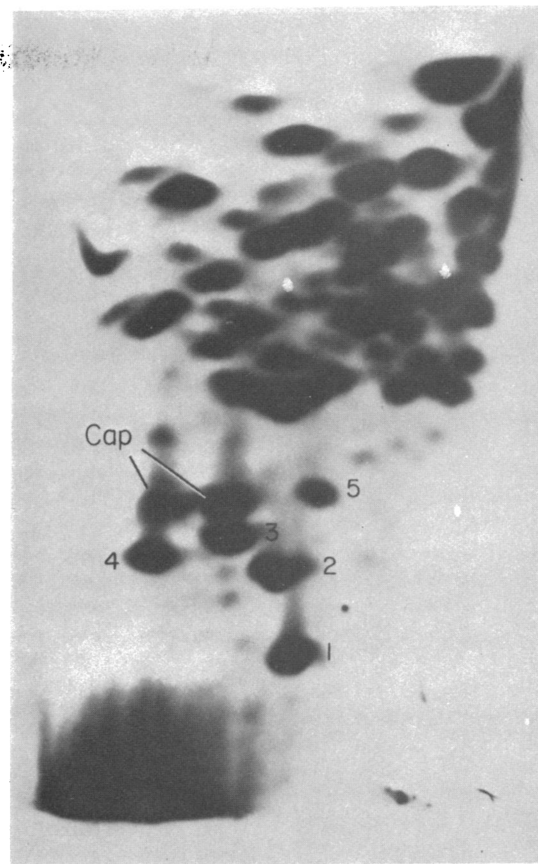

FIG. 2. T1 ribonuclease fingerprint analysis of DRB-resistant *Sma*-F-specific nuclear RNA. DRB-resistant RNA that hybridized to *Sma*-F was treated with RNase T1 and, after treatment with iodoacetic acid to inhibit RNase T1, the hybridized RNA was eluted from the filter, digested with T1, and fingerprinted as described by Fraser and Ziff (22). Separation in the first dimension (left to right) was on cellulose acetate in pH 3.5 pyridine/acetate buffer and 7 M urea. After transfer to thin-layer plates of DEAE-cellulose, homochromatography was performed in the second dimension (bottom to top).

RNA (Fig. 2). Further secondary analysis on the large T1 oligonucleotides (spots 1–5 in Fig. 2) after ribonuclease A digestion revealed products that were identical to those identified by Ziff and Evans (12). The two cap spots from the two-dimensional fingerprint were shown to contain oligonucleotides resistant to digestion by both RNase A and RNase T2. RNA specific for *11.1–18.2* eluted from gels similar to Fig. 1 also contained caps.

A second experiment was then performed so that DRB-treated and control samples could be compared at the same time. Short *11.1–18.2*-specific RNA was selected both without and with T1 RNase treatment after hybridization. This treatment should have removed any nucleotides that derived from transcription to the right of *18.2*. All five large oligonucleotides (1–5, Fig. 2) remained visible in all of the four fingerprints (Fig. 3). Furthermore, a large oligonucleotide in the position of the 5′ capped oligonucleotide was also seen in every case. A striking feature of the hybridized short RNA from untreated cells that was not "trimmed" with the RNase T1 (Fig. 3*B*) was the presence of extra sequences (a greater "complexity" of the finger-

fragments from Ad-2. The hybridized RNA was eluted, precipitated with ethanol, and dissolved in 10 μl of 10 mM Tris·HCl (pH 7.6)/10 mM EDTA. A 10-μl portion of formamide was then added and the mixture was heated to 95°C for 2 min. After cooling on ice and addition of bromophenol blue/glycerol, the sample was loaded onto a 5% polyacrylamide gel containing 20:1 acrylamide/bisacrylamide. The gel was run in half-strength Loening buffer containing 0.2% sodium dodecyl sulfate (24). Reserve buffer was recycled and the electrophoresis was continued until the bromophenol blue was ≈2 cm from the bottom. The figure represents a composite of autoradiographs from two separate experiments. Lanes 7 and 8 represent longer exposures of lanes 5 and 6.

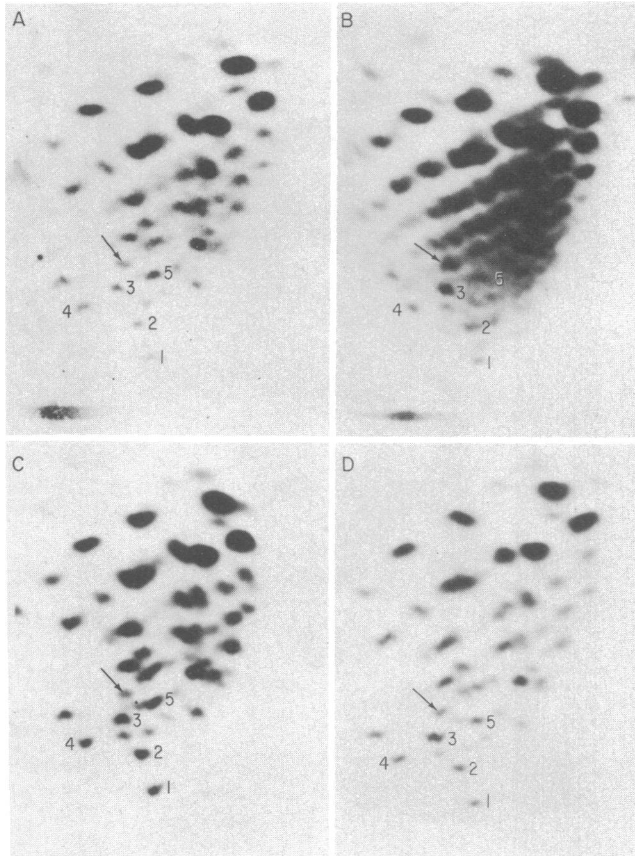

FIG. 3. T1 ribonuclease fingerprint analysis of *Sma*-F-specific small (4–14S) nuclear RNA from control, DRB-free, and DRB-treated cells. ^{32}P-Labeled small (4–14S) nuclear late Ad-2 RNA was hybridized to *Sma*-F DNA (*11.1–18.2*) bound to nitrocellulose filters. Duplicate filters were used for control (*A* and *B*) and DRB-treated samples (*C* and *D*). One of each duplicate was treated with RNase T1 on the filter as described in the legend to Fig. 2 (*A* and *C*). After elution from the filter the RNA was precipitated with ethanol and fingerprinted as described for Fig. 2. (*A*) Control, T1-trimmed on the filter; (*B*) control, untrimmed on the filter; (*C*) DRB, T1-trimmed on the filter; (*D*) DRB, untrimmed on the filter. The arrows point to capped oligonucleotides as judged by comparison with Fig. 2 and with fingerprints published by Ziff and Evans (12).

print) compared to the sample that had been treated with T1 (Fig. 3*A*). These extra sequences were not present in the hybridized, non-RNase-treated sample from DRB-treated cells (Fig. 3*D*). Therefore exposure of cells to DRB appears to have limited transcription of most RNA chains to sites within the *16.4–18.2* region.

By comparison with the fingerprint and sequence data reported by Ziff and Evans (12), we conclude that the major 5′ end in short nuclear *Sma*-F-specific RNA from both control and DRB-treated cells is the correctly capped 5′-oligonucleotide corresponding to the major, late Ad-2 promoter. Multiple RNA bands would be generated if RNA polymerases reading rightward from this point terminated synthesis at multiple discrete sites in the *16.4–18.2* region.

The early to late transition in *16.4* → *99*

The experiments describing prematurely terminated chains in the *16–20* region of the Ad-2 genome reported in this and other papers (5, 14, 15) have been performed with cells late in infection. During the course of infection with Ad-2 there is an early phase of RNA synthesis during which about 0.5–1% of total RNA output is virus specific, whereas late in infection this fraction is increased to as much as 30% (3, 29, 30).

The presence late in infection of premature transcripts from the *16–20* region of the Ad-2 genome might suggest an early to late regulation of Ad-2 RNA synthesis modeled on bacterial attenuation. In *Escherichia coli*, for example, when tryptophan is plentiful essentially *no* transcripts are elongated past a site within ≈200 nucleotides of the promoter of the tryptophan operon [the collection of genes responsible for enzymes that synthesize tryptophan (31, 32)]. When tryptophan is needed a *partial* relief of premature termination allows mRNA to be formed distal to the site of attenuation and tryptophan synthetic enzymes are formed. We wished to determine whether the premature rightward-reading transcripts complementary to the *16* to ≈*20* region of Ad-2 DNA were being made at equal rates at all times of infection. Two issues complicate the interpretation of a simple analysis of the amount of RNA hybridizable to the *16–20* region in pulse-labeled RNA samples produced early and late in infection. First the mRNA for the IVa^2 protein is read from *15* to *11* in a leftward direction within 5 hr after infection (20, 33, 34). Second, the number of potential templates rises greatly after Ad-2 DNA synthesis begins so that both a shut-down of early transcription and the availability of new templates could mask the possibility that premature termination in the *16–20* region was the effective means of preventing late mRNAs from appearing early. We have at least partially solved these problems by (*i*) measuring the hybridization of pulse-labeled RNA to separated strands in the *11.1–18.2* region and (*ii*) comparing the relative rates of RNA synthesis in the *11.1–18.2* rightward direction to neighboring rightward-reading transcribed regions within *0–11*, which regions are transcribed both early and late in infection (5, 14, 15, 20).

First, it was found that only about 35% of the transcription of the *11.1–18.2* region was rightward reading early in infection, whereas late in infection 93% of the transcription from this region is rightward reading (Table 1). Second, the ratio of synthesis in pulse-labeled RNA, presumably representing mainly nascent chains and therefore total rates of synthesis, from *3–10.7* compared to *11.1–18.2* early in infection was at least 2:1 and only 1:4 or 1:5 late in infection (Table 1). Corrected for the leftward-reading RNA at both times, this ratio indicates at least a 30-fold greater rightward synthesis late compared to early in infection for the *11.1–18.2* region.

Thus, the simplest possible attenuation control, namely unregulated initiation of transcription of *11.1–18.2* throughout infection but complete attenuation early in infection and partial relief of attenuation late in infection is not the basis of the early-to-late transition in Ad-2 RNA formation. Rather there is greatly increased RNA synthesis, including increased synthesis of prematurely terminated chains, beginning late in infection at the *16.4* site.

DISCUSSION

Previous studies had demonstrated that pulse-labeled RNA, whether produced *in vivo* or *in vitro*, contained at least 4-fold more labeled RNA complementary to the region *11* to ≈*20* on the Ad-2 genome than to any other region encompassed by the large late transcription unit that is read rightward from *16.4* to ≈*99* on the genome. These results signify that RNA chains are frequently started but not finished—i.e., prematurely terminated (5, 14, 15). To study the start sites and oligonucleotide compositions of these premature chains we were forced to label RNA *in vivo* because *in vitro* initiation of polymerase II products at a significant rate has yet to be accomplished. We have found that a great number of discrete bands complementary to the DNA region containing the start sites can be recovered. Moreover, in a T1 digest this RNA contains the capped oligonucleotides and other large diagnostic oligonucleotides of the first ≈80 to ≈200 nucleotides in approximately

Table 1. Early and late rightward transcription from the transcription units in the left-hand end of the Ad-2 genome

	Exp.	cpm hybridized to			% of *11.1–18.2* (rightward)	Ratio	
		0–3.0	*3.0–10.7*	*11.1–18.2*		*11.1–18.2* / *3.0–10.7*	*11.1–18.2*(r) / *3.0–10.7*
Early	1*	2920	12,150	5760	33.3	0.48	0.16
	2*	3414	11,530	1360	35.8	0.12	0.04
Late	1†	ND	1,519	9160	92.8	6.3	5.6
	2‡	195	740	2680	ND	3.6	3.3
	3§	ND	240	1480	ND	6.3	5.9

ND, not done.

* From ref. 20, 10-min [^{3}H]uridine pulse labels. Percentage rightward reading in column six was determined in Exps. 1 and 2.

† From ref. 14, 2-min [^{3}H]uridine pulse labels.

‡ Forty-five-second [^{3}H]uridine pulse label of cells 18 hr after infection.

§ Two-minute *in vitro* [^{3}H]UTP incorporation by nuclei isolated 16 hr after infection (5).

equal abundance. While the capped oligonucleotide is spliced into the Ad-2 mRNAs (9–12, 21), the other diagnostic oligonucleotides from the *16.4–18.2* region never reach the cytoplasm. However, all the oligonucleotides of the *16.4–18.2* region, including the cap, appear to be present in approximately equal amounts, suggesting that the accumulated discrete-sized short chains reported here are not residual products of RNA processing.

Another possible means of obtaining multiple RNA bands after autoradiography of nucleic acid products is the existence of "pause sites" for the polymerase (35). However, if the final result of such transcription is a pause followed by a continuation, then *equal* synthesis of the entire chain should result and be detected in very brief labels as equimolar synthesis. As stated above, in the case of the major late Ad-2 transcription unit it has been established (5, 14, 15) that RNA from the beginning of the transcriptional unit is *over-produced*, signifying premature termination. We therefore conclude that the bands we have examined in this paper most likely represent discrete premature termination products.

In agreement with our earlier suggestion (14, 20), the mode of action of DRB seems clearly to be an enhancement of premature RNA chain termination. The bands of the promoter-proximal RNA observed by radioautography were more intense in RNA from DRB-treated cultures than in untreated cultures. In addition, extra sequences extending beyond the *11–18* region in RNA from control cultures were not present in RNA from DRB-treated cultures. A normal mechanism that already causes frequent premature terminations appears to be increased by DRB to a point at which essentially no complete transcripts (and therefore no mRNA) can be made.

Finally, as will be discussed more completely elsewhere, the presence of caps in nuclear RNA molecules that are never completed and therefore cannot yield mRNA indicates that the addition of a cap to an RNA chain does not ensure production of an mRNA molecule (unpublished).

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